analytical chemistry

Article

pubs.acs.org/ac

Quantification, Localization, and Speciation of Selenium in Seeds of Canola and Two Mustard Species Compared to Seed-Meals Produced by Hydraulic Press

Gary S. Bañuelos,*[,†,‡] Spencer S. Walse,[†] Soo In Yang,[§] Ingrid J. Pickering,[§] Sirine C. Fakra,[∥] Matthew A. Marcus,[∥] and John L. Freeman*[,⊥]

[†]Agricultural Research Service, U.S. Department of Agriculture, Parlier, California 93648, United States

[‡]Center for Irrigation Technology, California State University Fresno, Fresno, California 93740, United States

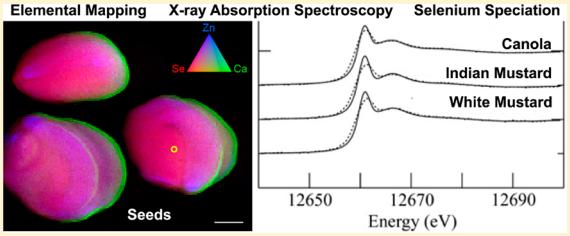
[§]Department of Geological Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E2, Canada

[∥]Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

[⊥]Department of Biology, California State University Fresno, Fresno, California 93740, United States

Supporting Information

ABSTRACT: *Brassica* plants accumulate selenium (Se) especially in seeds when grown in soils laden with Se. We report a chemical analysis of Se in *Brassica* seeds (canola, Indian mustard, and white mustard) and in their hydraulically pressed seed meals, which are used as a Se supplement in livestock animal feeds. Complementary techniques were used to measure total Se concentrations, to map the localization of Se, and to quantify different Se forms. Seeds and hydraulically pressed seed meals contained an average of 1.8 and 2.0 μg Se g^{-1} DW, respectively. Selenium was primarily located in cotyledons and roots of seed embryos. Microfocused Se K-edge XANES and bulk XANES showed that seeds contained 90% of Se as C−Se−C forms. Hydraulically pressing seeds for oil caused changes in the forms of Se as follows: 40−55% C−Se−C forms, 33−42% selenocystine, 5−12% selenocysteine, and 11−14% trimethylselenonium ion. Aqueous extracts of seed and seed meals were also analyzed by SAX-HPLC/ICPMS and found to contain mainly the C−Se−C form SeMet, but also another C−Se−C form MeSeCys, which is of dietary pharmacological interest for cancer inhibition. In addition, SAX-HPLC/ICPMS also detected selenocystine and selenocysteine, further confirming the results obtained by XANES analyses.



Soils located on the western side of California's San Joaquin Valley (WSJV) are naturally laden with inorganic selenium (Se) oxides, primarily selenate (SeO_4^{2-}), which is soluble and can accumulate in aquatic systems to levels that are toxic to a variety of organisms.[1] Bañuelos and co-workers previously adopted a phytoremediation strategy to remove SeO_4^{2-} from these soils through uptake into plant shoots of several *Brassica* species, canola (*Brassica napus*), Indian mustard (*Brassica juncea*), and white mustard (*Sinapis alba*) that are being utilized on an agricultural-scale to manage soil Se.[2] In general, *Brassica* species uptake and convert inorganic SeO_4^{2-} into multiple organic storage forms of Se, some of which are volatilized from leaves into the air.[3] Plants containing organic forms of Se can then be harvested and/or processed into a variety of consumables that afford nutritional benefit.[2] For example, *Brassica* seeds are pressed to recover oil for biodiesel production (>35% oil by seed DW), and the resulting Se-enriched seed meals are used to supplement livestock feed with bioavailable Se that efficiently provides this essential mineral requirement to animal diets.[4,5] New analytical methods are being developed and integrated to help establish a more complete profile of the Se forms within plants used for phytoremediation and in the subsequent plant derived Se-enriched biofortified feed products.[3,6−11]

In this study, we combined a complementary suite of analytical methods to measure total Se content and its forms within seeds and hydraulically pressed seed meals. Specifically, the concentrations of total Se were measured, the localization of Se was determined, and the chemical forms of Se were quantified using inductively coupled plasma mass spectrometry (ICPMS), micro X-ray fluorescence (μ-XRF) mapping, micro and bulk X-ray absorption near edge structure (XANES) spectroscopy, and strong anion exchange-high performance liquid chromatography coupled to ICPMS (SAX-HPLC/ICPMS).

EXPERIMENTAL SECTION

Instrumentation. *ICPMS.* An Agilent 7500cx ICPMS was used to determine total Se concentrations (Agilent Technologies, Santa Clara, CA). The National Institute of Standards

Received: March 23, 2012
Accepted: June 29, 2012

   dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX−XXX

and Technology (NIST) wheat flour (SRM 1567; Se content of 1.1 ± 0.2 μg g^{-1} DW) was used as the Se quality control standard for plant analysis with a > 94% recovery as described in ref 12.

μ-XRF/μ-XANES. Microprobe analyses were done on beamline 10.3.2., Advanced Light Source Lawrence Berkeley National Laboratory (ALS-LBL). Frozen seeds were transferred onto a Peltier stage at −33 °C to minimize radiation damage. Microfocused X-ray fluorescence (μXRF) elemental mapping was used to characterize the localization of Se in whole seeds, and microfocused Se K-edge X-ray absorption near edge structure (μXANES) spectroscopy was performed in the most concentrated areas of Se to determine the forms of Se. Maps were recorded at 13 000 eV, using a 15 μm (H) × 6 μm (V) beam spot size, a 15 μm × 15 μm pixel size, and 50 ms dwell time per pixel. Maps and XANES spectra were recorded with a 7 element Ge solid state detector (Canberra Industries Inc., Meriden, CT). Selenium XANES spectra were dead-time corrected, pre-edge background subtracted, and post-edge normalized using standard procedures.[7] Red Se (white line maximum set at 12660 eV) was used to calibrate the spectra. Least square linear combination fitting (LCF) of the XANES spectra was performed in the 12 630−12 850 eV range, using a library of nine standard selenium compounds listed in reagents and standards. The error on the percentages of species present is estimated to be ±10%. All data processing and analyses were performed with a suite of custom LabVIEW programs at the beamline.

Bulk XANES. Spectra were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) with the storage ring operating at 3 GeV. Selenium K-edge spectra were recorded on beamline 9-3 with an upstream Rh-coated collimating mirror, a Si(220) double-crystal monochromator, and a downstream Rh-coated focusing mirror. The incident X-ray intensity was monitored using a N_2-filled ionization chamber. XANES spectra were recorded by monitoring the Se Kα fluorescence using a 30 element germanium detector equipped with Soller slits and an arsenic filter. During data collection, samples were maintained at approximately 10 K in a liquid helium flow cryostat.[10] Fluorescence spectra were also collected on dilute aqueous solutions of standard Se species buffered at pH 7, including the inorganic Se forms as SeO_4^{2-} and selenite (SeO_3^{2-}), and the organic forms of selenomethionine (SeMet), trimethylselenonium ion ($TMSe^+$), selenocystine (CysSeSeCys), selenocysteine ($SeCys^-$), and selenodiglutathione (GSHSeGSH). Solid red elemental Se was also measured in transmission mode. Sample spectra were calibrated with respect to the spectrum of hexagonal Se placed downstream of the sample and measured simultaneously in transmission, the first energy inflection of set at 12 658 eV. Background subtraction, normalization, and data analyses were carried out according to standard procedures using the EXAFSPAK program suite. XANES spectra were analyzed by least-squares LCF.[9] The Se XANES spectrum of a sample containing multiple Se species is a weighted sum of the spectra from the species present in the sample. The spectra were analyzed by least-squares linear combination fitting to extract the percentage contribution from each species (assuming that all species present were represented in the set of standards). The percentage contribution of a standard spectrum to the sum is equal to the percentage of total Se species present in that chemical form.

SAX-HPLC/ICPMS. Per manufacturer instructions and the Agilent selenium speciation protocol, an Agilent 1200 HPLC separations module equipped with a Hamilton PRPX-100 strong anion exchange analytical column (10 μm particle size-25 cm length × 4.1 mm internal diameter) and an Agilent 7500 ICPMS were used for analysis of Se forms in aqueous extracts.[11] A single analysis (30 μL injection) was conducted for each of the aqueous extracts. Chromatographic separation of aqueous Se was achieved with an isocratic mobile phase of 5 mM ammonium citrate buffer (pH 5.2) with 2% methanol at a flow rate of 1 mL/min. Retention times of ^{78}Se containing peaks were monitored using the ICPMS and directly compared to the authentic standards (listed below) that were analyzed after spiking into a blank methanol chloroform water (MCW) extraction as described by.[6]

Reagents and Standards. All reagents were analytical grade and were used without purification. Samples were acid digested for total Se quantification using HNO_3, H_2O_2, and HCl and an automatic temperature-controlled heat block as described in ref 12. All solutions used in analyses were prepared in 18 MΩ cm doubly deionized water generated by a Milli-Q Plus treatment system (Millipore, Molsheim, France). Aqueous solutions of the following selenium compounds were used as standard materials for making μ-XANES spectral fit and SAX-HPLC/ICPMS comparisons: SeO_4^{2-} and SeO_3^{2-}, SeMet, CysSeSeCys (all purchased from Sigma-Aldrich, St. Louis, MO). Methyl-selenocysteine (MeSeCys), selenocystathionine, γ-glutamyl-methyl-selenocysteine (γGluMeSeCys), and selenodiglutathione (GSHSeGSH) were all purchased from PharmaSe (www.pharmaSe.com), while red and gray elemental Se (Se^0) samples were a gift from Dan Strawn at ALS-LBL. Selenocysteine used for XANES was obtained by reducing selenocystine at 70 °C overnight in 100 mM sodium borohydride at a 1:1 molar ratio. However, selenocysteine used for SAX-HPLC/ICPMS was obtained by reducing selenocystine at 70 °C overnight in 50 mM dithiothreitol (DTT) at a 1:1 molar ratio. For EXAFS and bulk XANES, fluorescence spectra were collected on dilute aqueous solutions of standard Se species buffered at pH 7, including the inorganic Se forms as SeO_4^{2-} and SeO_3^{2-}, the organic forms of SeMet, trimethylselenonium ion $TMSe^+$, CysSeSeCys, $SeCys^-$, and GSHSeGSH. Red elemental Se was also measured in transmittance. Protease XIV, methanol (ultrapure), chloroform, Waters Sep-Pak Classic C18 cartridges (360 mg 55−105 μm), 88% formic acid, and Agilent screw-top glass HPLC vials were used for sample preparation prior to SAX-HPLC/ICPMS, and an isocratic mobile phase consisting of 5 mM ammonium citrate buffer (pH 5.2) with 2% methanol was used for chromatographic separation as per Agilent manufacturers instruction and protocol.[11]

Plant Growth, Hydraulic Seed Pressing, and Sample Preparation. Canola (*Brassica napus* var. Hyola 420), Indian mustard (*Brassica juncea* var. Pacific Gold), and white mustard (*Sinapis alba* var. Ida Gold) seeds were purchased from commercial seed suppliers. In order to perform micro XANES, growth chamber planted seeds were grown in a sandy loam soil (coarse-loamy, mixed superactive, nonacid, thermic Typic Xerothent) and were watered three times weekly with 20 μM SeO_4^{2-}. Plants were grown at 24 °C/20 °C, 10 h/14 h light/dark, and 140 μmol m^{-2} s^{-1} photosynthetic photon flux. Separately, two 50 ha field sites located on the WSJV were, respectively, planted with each of the above *Brassica* seeds in a oxalis silty clay loam soil with a sodium sulfate-dominated

dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX−XXX

salinity ranging from 5 to 8 dS m^{-1} and having a water-soluble Se content ranging from 0.1 to 0.15 mg L^{-1}. With 180 days after planting, all seeds had dried naturally on the plants and were harvested. Seeds from growth chamber- and field-grown plants were then flash frozen in liquid nitrogen and stored at −80 °C. A model 2000 Insta Pro Extruder (125 HP, turbo charged diesel main drive motor, Des Moines Iowa) and a model 1500 Insta Pro Continuous Horizontal Press (50 HP, Des Moines, Iowa) were used to produce seed meals after mechanically removing oil from *Brassica* field-grown seeds. Due to the friction and pressure caused by pressing the seeds through a series of incrementally decreasing pore sizes, the extruder heats up seed meals to a range 83−105 °C. The *Brassica* seed slurry is then put through a secondary horizontal press, which further ruptures cells and captures the remaining oil, while separating it from the residual seed meal.

For microprobe analyses, whole seeds were rinsed with 18 MΩ cm doubly deionized water, blotted dry with paper towels, flash-frozen in liquid nitrogen, and taken to the LBL-ALS beamline 10.3.2.

For bulk XANES, whole seeds and seed meals were both finely ground using a mortar and pestle in liquid nitrogen, packed into 2 mm path length sample cells, and maintained in liquid nitrogen for Se K-edge XANES over the homogenously mixed finely ground sample.

Sample Extraction SAX-HPLC/ICPMS. Selenium compounds were extracted using a methanol, chloroform, and water (MCW) extraction,[6] to isolate 60% MeOH soluble Se compounds in seeds and seed meals for subsequent characterization. Duplicate seed or seed meal samples (1.0−1.5 g) were placed in separate 40 mL glass vials equipped with Teflon caps. Protease XIV (50 mg) and water (10 mL) were added to one vial. The other vial received 17 mL of methanol (ultrapure) without protease or water. The paired samples were vortexed, and the protease sample set was incubated in a shaker for 20 h at 37 °C, while the sample set with methanol was maintained overnight at 4 °C. Methanol (17 mL) was then added to the protease-digested samples, and water (10 mL) was added to the non-protease-digested samples. All samples were mixed thoroughly and refrigerated overnight at 4 °C. Chloroform (8.5 mL) was then added to all vials, which were shaken vigorously, and then refrigerated again at 4 °C overnight. Vials were tapped to break emulsions between the upper aqueous (methanol−water) phase and the lower organic phase. The aqueous phase (∼27 mL of 60% MeOH) was then carefully transferred with a Pasteur pipet to a 50 mL conical centrifuge tube. One quarter of the aqueous phase (∼6.8 mL) was then pipetted into a 50 mL ICP digestion tube, evaporated with a heating block at 50 °C (∼300 min), acid digested,[12] and analyzed for Se by ICPMS. The nonaqueous chloroform phase remaining in the original 40 mL glass vial was also evaporated with a heating block at 50 °C (∼300 min), acid digested,[12] and analyzed for Se by ICPMS. The percentage of Se in the aqueous phase after extraction was calculated as 100 × (total Se in aqueous phase)/[(total Se in aqueous phase) + (total Se in chloroform)]. The remaining aqueous phase (∼20.3 mL) was dried *in vacuo* with a refrigerated centrifugal speed vacuum, resuspended in 2.5 mL water, and stored at −80 °C. Waters Sep-Pak Classic C18 cartridges (360 mg 55−105 μm) were used for final cleanup of the aqueous concentrates. Each cartridge was cleaned by flushing 10 mL of methanol and 5 mL of ultrapure water in succession. Each frozen sample concentrate in 2.5 mL water was thawed and vortexed; formic acid (88%; 11 μL) was added before the sample was transferred with a Pasteur pipet to the cartridge and the sample was eluted using the syringe into a new 50 mL conical tube. Methanol (3 mL) was then used to elute any residual into the same 50 mL conical tube. Eluant was then dried *in vacuo* as above, dissolved in water (1.5 mL), transferred into Agilent screw-top glass HPLC vials, and stored frozen (−80 °C) until SAX-HPLC/ICPMS analysis.

RESULTS AND DISCUSSION

Total Selenium. Concentrations of total Se were measured by ICPMS in both field-grown Brassica seeds and seed meals, which generally exhibited similar mean Se concentrations ranging from 2.14 ± 0.75 to 1.36 ± 0.33 in seeds and from 2.44 ± 0.61 to 1.57 ± 0.23 in seed meals [$x \pm s$ (SD), μg Se g^{-1} dry weight (DW)], respectively (Figure 1). Less than 1% of the other elements measured were removed from seed meals during two consecutive hydraulic oil presses (Table S-1.). These results indicated that the hydraulic press process does not decrease the total mineral concentrations (including Se) of seeds after hydraulically pressing into seed meal.

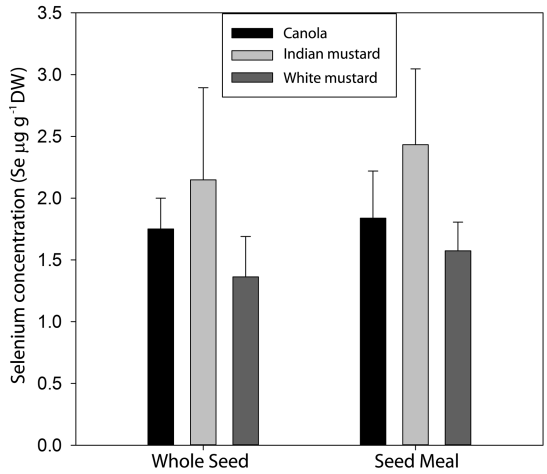


Figure 1. Mean concentrations of total Se in seeds and seed meals of WSJV field grown canola (coded in black), Indian mustard (coded in light gray), and white mustard (coded in dark gray). Error bars represent standard deviations, $n = 3$ measurements per each sample. No statistical difference in mean Se concentration was observed between any sample at a p-value ≤ 0.05.

Selenium Distribution. Micro-XRF elemental mapping showed the spatial distribution of Se (coded in red), Ca (coded in green), and Zn (coded in blue), present in whole seeds harvested from canola (Figure 2A) and Indian and white mustards (Figure 2B,C, respectively) after feeding plants 20 μM SeO_4^{2-} under growth chamber conditions. These chamber grown seeds had accumulated a range of Se concentrations between 100 and 200 μg Se g^{-1} DW. As part of the main focus of this study, Indian and white mustard seeds collected from the WSJV field-grown plants are shown for comparison (Figure 2D,E, respectively). The Se, Ca, and Zn maps of growth chamber- and field-grown seeds had the same elemental localization patterns. In the seeds, Se was mainly localized in cotyledons and roots of the embryo, while Zn was mainly localized in root tips, and Ca was primarily observed in the seed coat. The micro-XRF elemental mapping penetration depth for Se is 1.4 mm or through the entire seed; however, the Ca map

dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX−XXX

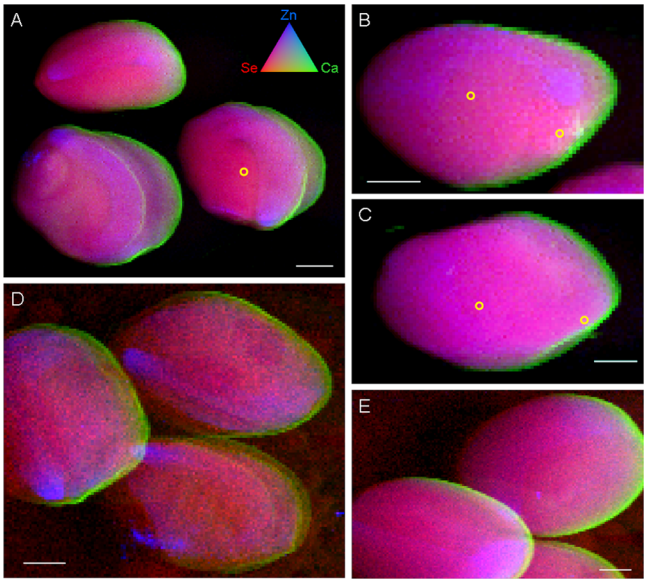


Figure 2. Distribution of Se in canola and Indian and white mustard seeds. Microfocused X-ray fluorescence (μXRF) maps show the spatial distribution of Se (in red), Ca (in green), and Zn (in blue), in canola (A), Indian mustard (B), white mustard (C) fed Se while growing in greenhouse, and Indian mustard (D) and white mustard (E) grown in WSJV field. Scale bars = 800 μm (A), 1 mm (B and C), 700 μm (D), and 900 μm (E). Yellow circles (A–D) show locations of micro X-ray absorption near edge structure (μXANES) spectra reported in Table S-2.

is somewhat limited because the X-ray penetration depth for the detection of Ca is no more than about 100 μm. While all three minerals mapped are nutritionally important to livestock and humans, Zn and Ca were included for multiple reasons. Zinc in direct comparison to Se was important because we have recently observed that Se and Zn localizations are often inversely correlated in Brassica seeds. Calcium is included because it was accumulated in the seed coat, while Se and Zn were not. Furthermore, by overlaying all three elements the anatomical structures of the seeds are visible, including the seed coat, embryo cotyledons, root, and root tip, while it is of major future biological interest that Zn was accumulated primarily in embryo root tips of these Brassica seeds.

Micro XANES. Se K-edge μXANES spectroscopy was used to determine the chemical forms of Se in specific areas (denoted by yellow circles) of canola and Indian and white mustard seeds harvested from plants that received 20 μM SeO_4^{2-} in the growth chamber. Least square linear combination (LC) fitting of μXANES spectra from these seeds was performed using a library of standard Se compounds. Spectral fitting results demonstrated that canola and Indian and white mustard seed all contained ≥99 ± 10% C–Se–C forms (Table S-2). Micro-XANES is sensitive to the local bond geometry and electronic structure of the central Se atom and therefore cannot easily distinguish between C–Se–C compounds such as SeMet, MeSeCys, or γGluMeSeCys. Microfocused XANES showed that SeO_4^{2-}, SeO_3^{2-}, $SeCys^-$, Cys–Se–Se–Cys, Se^0, and RS–Se–SR were not detectable. An advantage of using Se K-edge μ-XANES is it allows one to directly probe the system and the results are not prone to potential artifacts of liquid extraction. A hindrance of Se K-edge μ-XANES analysis is in the detection limits for Se, and indeed, the Se concentrations in seeds harvested from WSJV field-grown plants were below μ-XANES detection.

Bulk XANES. Bulk XANES Se K-edge analysis also allows one to directly probe the entire sample, which is finely ground and held in a 2 mm cuvette placed in a liquid nitrogen filled cryostat. In bulk XANES the penetration depth for the straight Se K line is 3.2 mm; hence, the X-rays penetrate the entire biological sample before going into the detector located directly in line behind the sample, and because the original parent materials are analyzed, the results are not prone to potential artifacts of liquid extraction or processing. The detection limits are much lower than μ-XANES, and thus, bulk XANES showed that the WSJV field-grown *Brassica* seeds contained between 90–95% C–Se–C forms and 5–10% seleno-diglutathione (Figure 3, Table 1). Importantly, the bulk XANES analysis of

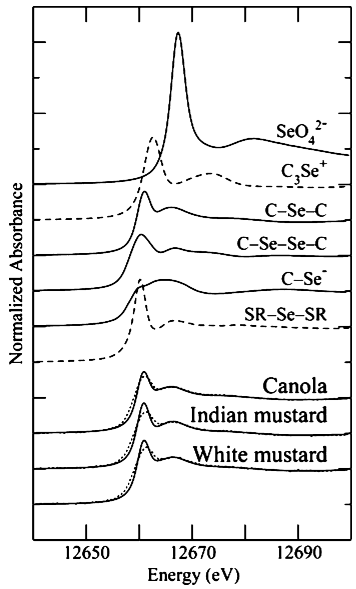


Figure 3. Normalized Se K near-edge spectra of WSJV field-grown *Brassica* seed and seed meals. Spectra of canola and Indian mustard and white mustard whole seeds (—), and the respective seed meals after oil extraction (···), are compared. In reference to those Se species found in the samples, these standards spectra are shown as follows: SeO_4^{2-} (selenate), C_3Se^+ (trimethylselenonium ion), C–Se–C (selenomethionine), C–Se–Se-C (selenocystine), C–Se$^-$ (selenocysteine), SR–Se–SR (selenodiglutathione). The tick marks on the *Y*-axis show relative absorbance. For clarity, C_3Se^+ and SR–Se–SR spectra are shown as broken lines.

all three seed meals showed a shift in the amount of C–Se–C forms which decreased to only 40–55% and demonstrated the appearance of 5–12% selenocysteine, 33–42% selenocystine, and 0–14% trimethylselenonium ion (Table 1).

The shift in C–Se–C forms, likely SeMet, may be caused by heat and pressure encountered during the hydraulic pressing for seed oil and is apparently followed by the subsequent oxidation of reduced seleno-cysteine (C–Se) which is readily oxidized through contact with oxygen in the air and water, into selenocystine (C–Se–Se–C). Using Se K-edge to identify selenocystine (C–Se–Se–C) as a major form in the seed meals is important because of its low solubility and thus difficult identification by liquid chromatography. These results demonstrate that it is of major importance to investigate the chemical forms of Se before and after food processing and to use Se K-edge analyses because the chemical forms of Se can readily

dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX–XXX

Table 1. Forms of Se as a Percentage of Total Se by Bulk X-ray Absorption Near-Edge Structure (XANES) of Se in *Brassica* Seeds and Seed Meals[a]

plant	seed part	C−Se−C	C−Se−Se−C	CSe^-	RS−Se−SR	C_3Se^+	SeO_4^{2-}
canola	whole ground seed	90 (1)			10 (1)		
	seed meal	41 (4)	35 (4)	8 (5)		14 (2)	2 (1)
Indian mustard	whole ground seed	95 (2)			5 (2)		
	seed meal	55 (3)	33 (4)	12 (4)			
white mustard	whole ground seed	90 (1)			10 (1)		
	seed meal	40 (4)	42 (4)	5 (5)		11 (2)	2 (1)

[a]Least-square linear combination fitting of bulk selenium K-edge XANES spectra. Observed selenium species include the following: C−Se−Se-C, modeled as selenocystine (CysSeSeCys), $C-Se^-$ (selenocysteine, $SeCys^-$), RS−Se−SR (selenodiglutathione, GSHSeGSH), C_3Se^+ (trimethylselenonium ion, $TMSe^+$), selenate (SeO_4^{2-}), and R−Se−R (selenomethionine, SeMet). Selenite (SeO_3^{2-}) and elemental selenium (Se^0) were not detected. Three times-estimated standard deviations are shown in the parentheses after the observed percent of each Se species.

change from those found in parent biological materials such as in these seeds.

SAX-HPLC/ICPMS. A major advantage to Agilent's SAX-HPLC/ICPMS manufacturers protocol for the speciation of organic and inorganic forms of Se is that it quantifies the Se atom directly and thus has the best sensitivity of all analytical methods typically used, and has a much lower detection limit compared to Se K-edge XANES analyses and conventional LC/MS. Furthermore, it has greater peak resolution for comparing both organic and inorganic Se compounds in a single run, which allows one to separate, identify, and quantify all the different forms of Se, including the various C−Se−C forms, which complement XANES analysis.

The percent of total Se recovered in the aqueous phase (60% methanol) after protease XIV-digestion of seed and seed meal extracts using MCW were, respectively, 34% and 29% for canola, 29% and 30% for Indian mustard, and 18% and 15% for white mustard. Protease XIV-digestion increased the aqueous phase recovery of Se in MCW extracts ~6% in seed and ~5% in seed meals. The total Se in the aqueous phase after protease XIV-digestion was ~33% of the total Se contained in seeds and meals. SAX-HPLC/ICPMS chromatographs of the aqueous Se compounds in protease XIV-digested seed and seed meals are shown in Figure 4. They were quantified as the mean percentage of aqueous Se ($x \pm s$, Table S-3). Starting with the first Se-containing peak the following results were obtained: canola seed (Figure 4 A, —) contained the aqueous Se species 14 ± 3% selenocystine (CysSeSeCys), 20 ± 2% MeSeCys, 66 ± 1% SeMet; Indian mustard seed (Figure 4 B, —) contained 17 ± 1% selenocystine (CysSeSeCys), 29 ± 2% MeSeCys, 31 ± 2% SeMet, 23 ± 2% $SeCys^-$; and white mustard seed (Figure 4 C, —) contained 15 ± 1% selenocystine (CysSeSeCys), 17 ± 1% MeSeCys, 34 ± 1% SeMet, 14 ± 1% $SeCys^-$, and 20 ± 4% selenate. The monomethylated form MeSeCys was found in whole seeds of canola (~20%), Indian mustard (~29%), and white mustard (~17%). MeSeCys was of particular interest since it reportedly has the highest rate of inhibiting cancer growth in cancer model animal systems.[13] Selenomethionine is also an important form of Se in crop plants and grains that is easily utilized by animal consumers and, due to its bioavailabilty, is often incorporated into proteins of meat, milk, whole blood, or serum (particularly in serum albumin).[3,14]

Using SAX-HPLC/ICPMS, we identified the forms of Se in hydraulically pressed seed meals to be different than the forms of Se found in seeds. Canola seed meal (Figure 4 A, ···) contained the following percentages of aqueous Se species: 7 ± 1% selenocystine (CysSeSeCys), 27 ± 6% MeSeCys, 55 ± 2% SeMet, 10 ± 3% $SeCys^-$ (Table S-3). Indian mustard seed meal (Figure 4 B, ···) contained 5 ± 1% selenocystine (CysSeSeCys), 20 ± 3% MeSeCys, 42 ± 5% SeMet, 32 ± 7% $SeCys^-$. White mustard seed meal (Figure 4 C, ···) contained 12 ± 1% selenocystine (CysSeSeCys), 38 ± 1% MeSeCys, 38 ± 3% SeMet, 18 ± 1% $SeCys^-$. The presence of 38% of total Se as MeSeCys in the white mustard (*Sinapis alba*) seed meal is of interest as a potential human food source for MeSeCys and is significantly higher than observed in the other meals. In addition to MeSeCys and SeMet, the presence of selenocystine (CysSeSeCys) and $SeCys^-$ in these seed meals is also of nutritional significance, since a livestock animal's health may be positively influenced by consuming a variety of organic Se forms. These organic Se forms may influence more than an animal's overall Se status, because a mixture of these forms should also increase enzymatic levels of Se dependent antioxidant enzymes, such as selenoglutathione peroxidase, which has $SeCys^-$ located at the active site.[3,14]

On the basis of the XANES data, there appears to be a change of SeMet into $SeCys^-$ and CysSeSeCys, and this observation suggests that the appearance of these latter two selenoamino acids may be due to the heat and pressure generated by the hydraulic oil press. The mixture of Se compounds that we observed in the *Brassica* seed meals may be of added nutritional value compared to livestock feed supplemented with Se-enriched wheat or a Se-enriched yeast, which are thought to contain mostly SeMet.

The presence of MeSeCys in the seed meals suggests that human food products such as granola bars or other food products, and animal feeds made with these Se-enriched *Brassica* seeds, in particular white mustard (*Sinapis alba*), may have added disease-preventative health benefits aside from providing the essential amounts of Se. MeSeCys is reportedly one of the least toxic forms of Se not readily misincorporated into proteins, and is reported to be one of the two most effective anticarcinogenic forms of Se.[3,13] While our finding that MeSeCys is present in *Brassica* seeds is the first of its kind, MeSeCys was previously detected in canola shoots at the following Se percentages: 11% $SeCys^-$, 14% CysSeSeCys, 32% MeSeCys, 43% SeMet.[4] It was also found in flowers of Indian mustard without protease digestion: 11% SeO_4^{2-} and 6% SeO_3^{2-}, 16% CysSeSeCys, and 67% MeSeCys.[15] These results all suggest that *Brassica* plants grown in the Se and Se-enriched soils of the WSJV are a good source for producing MeSeCys, a potent anticarcinogenic form of Se, for use in Se-enriched biofortified foods and animal feeds.

dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX−XXX

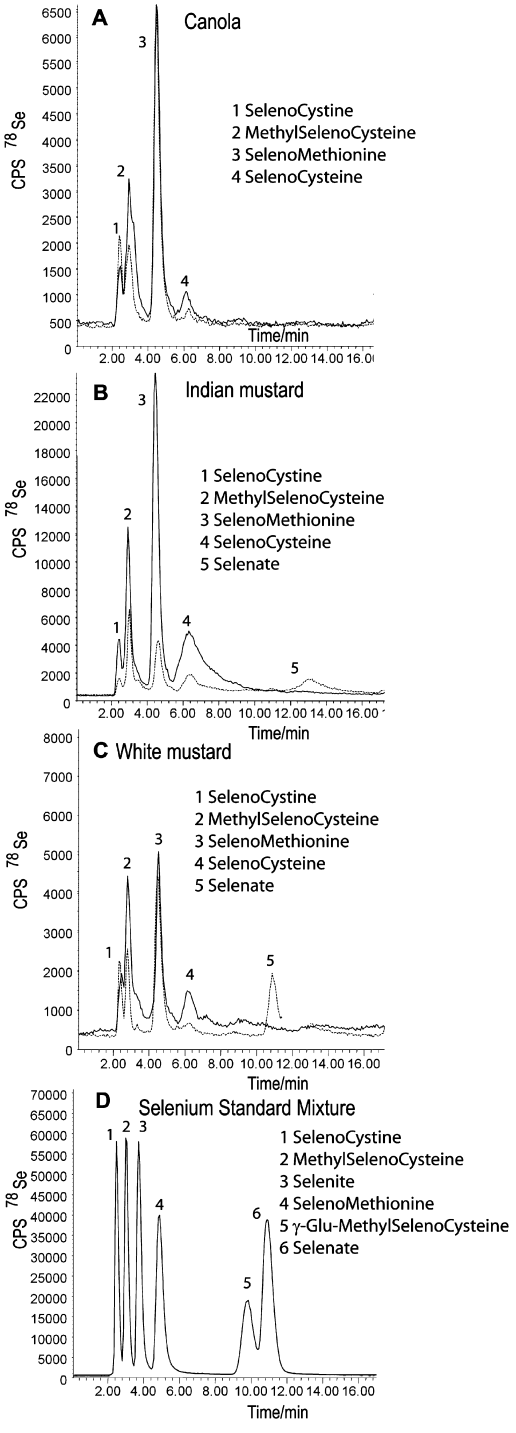


Figure 4. Chromatographic separation of the aqueous Se compounds present in canola (A), Indian mustard (B), and white mustard (C) whole seeds (—), and hydraulic pressed seed meals post oil extraction (···) after protease XIV-digestion using strong anion exchange-high performance liquid chromatography/inductively coupled plasma mass spectroscopy (SAX-HPLC/ICPMS). Selenium-containing peaks, monitored as ^{78}Se, are also shown for an MCW spiked Se standard mixture (D). Peaks are quantified as percentage of total ^{78}Se and presented in Table S-3. Standard additions were sequentially spiked into all samples to further verify peak identities, and Indian mustard sample is shown after this process as a representative in Supporting Information Figure 1.

CONCLUSIONS

The combined analytical techniques described herein represent the first reported study that has conjunctively utilized ICPMS, μXRF mapping, μXANES, bulk XANES, and SAX-HPLC/ICPMS for a comprehensive identification and quantification of Se in *Brassica* seeds and seed meal. Our observations demonstrate the importance for Se-biofortification in performing an accurate analysis of Se in livestock feed before and after processing. The complementary analyses of Se provide a comprehensive profile of the various forms of Se naturally present in the *Brassica* seeds and the changes of Se forms in the seed meals after processing with the hydraulic oil press. The results suggest that studies using only one type of Se analysis likely do not measure all the Se species present in a Se-biofortified feed product. The knowledge gained in this study may improve the nutritional health of humans and livestock animals through accurately providing them with known forms of Se in the mass-produced *Brassica* seed meals for use as a supplemental source of dietary Se. Furthermore, the combined use of these complementary methods will serve as a model for future studies and will help to ensure accurate identification of Se forms in biofortified foods and animal feeds before and after processing.

ASSOCIATED CONTENT

Supporting Information

Additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: JohnFreeman@csufresno.edu (J.L.F.), Gary.Banuelos@ars.usda.gov (G.S.B.). Fax: 559-596-2851. Web: http://www.ars.usda.gov/PandP/docs.htm?docid=22145.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Funding for this work was mainly provided by a CSU Fresno Agricultural Research Initiative and the California Department of Water Resources grant to G.S.B. at the USDA-ARS-WMR, SJVASC. We acknowledge and thank Irvin Arroyo and Annabel Rodriguez at the USDA-ARS in Parlier, CA, for help with sample preparation and ICPMS analyses. I.J.P. is a Canada Research Chair, and S.I.Y. is funded by the Natural Sciences and Engineering Research Council of Canada (Discovery Grant to I.J.P.) and by a CIHR-THRUST Fellowship. The Stanford Synchrotron Radiation Lightsource (SSRL) is a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program (P41RR001209). The operations of the Advanced Light Source at Lawrence Berkeley National Laboratory are supported by the Director, Office of Science, Office of Basic Energy Sciences, U.S. Department of Energy under Contract DE-AC02-05CH11231. G.S.B. and J.L.F. contributed equally to this work.

REFERENCES

(1) Ohlendorf, H. M.; Hoffman, D. J.; Saiki, M. K.; Aldrich, T. W. *Sci. Total Environ.* **1986**, *52*, 49−63.
(2) Bañuelos, G. S. In *Development and Uses of Biofortified Agricultural Products*; Bañuelos, G. S., Lin, Z. Q., Eds.; CRC Press, Taylor and Francis Group: Boca Raton, FL, 2009; pp 57−70.
(3) Zayed, A.; Pilon-Smits, E.; de Souza, M.; Lin, Z. Q.; Terry, N. In *Phytoremediation of Metal Contaminated Soil and Water*; Terry, N., Bañuelos, G.S., Eds.; CRC Lewis Publishers: New York, 2000; pp 61−38.
(4) Bañuelos, G. S.; Mayland, H. F. *Ecotoxicol. Environ. Saf.* **2000**, *46*, 322−328.
(5) Combs, G. F. *Biofactors* **2000**, *12*, 39−43.
(6) Bãnuelos, G. S.; Fakra, S. C.; Walse, S. S.; Marcus, M. A.; Yang, S.-I.; Pickering, I. J.; Pilon-Smits, E. A. H.; Freeman, J. L. *Plant Physiol.* **2011**, *155*, 315−327.
(7) Freeman, J. L.; Zhang, L. H.; Marcus, M. A.; Sirine Fakra, S.; McGrath, S. P.; Pilon-Smits, E. A. H. *Plant Physiol.* **2006**, *142*, 124−134.
(8) Kelly, S. D.; Hesterberg, D.; Ravel, B. In *Methods of Soil Analysis, Part 5—Mineralogical Methods*; Ulery, A. L., Drees, L. R., Eds.; Soil Science Society of America: Madison, WI, 2008; pp 367−464.
(9) Pickering, I. J.; George, G. N.; Van Fleet-Stalder, V.; Chasteen, T. G.; Prince, R. C. *JBIC, J. Biol. Inorg. Chem.* **1999**, *4*, 791−794.
(10) Andrahennadi, R. M.; Wayland, M.; Pickering, I. J. *Environ. Sci. Technol.* **2007**, *41*, 7683−7687.
(11) Bueno, M.; Pannier, F.; Potin-Gautier, M. *Agilent Technologies Inc.* **2007**, 5989−7073 , EN: 1−5.
(12) Bañuelos, G. S.; Akohoue, S. *Commun. Soil Sci. Plant Anal.* **1994**, *25*, 1655−1670.
(13) Ip, C.; Ganther, H. E. In *Cancer Chemoprevention*; Wattenberg, I., Lipkin, M., Boon, C. W., Kellott, G. J., Eds; CRC Press: Boca Raton, FL, 1992; pp 479−488.
(14) Whanger, P. D. *J. Am. Coll. Nutr.* **2002**, *21*, 223−232.
(15) Quinn, C. F.; Prins, C. N.; Freeman, J. L.; Gross, A. M.; Hantzis, R.; Laura, J.; Reynolds, J. B.; Yang, S-I.; Covey, P. A.; Bañuelos, G. S.; Pickering, I. J.; Fakra, S. C.; Marcus, M. A.; Arathi, H. S.; Pilon-Smits, E. A. H. *New Phytol.* **2011**, *192*, 727−737.

dx.doi.org/10.1021/ac300813e | *Anal. Chem.* XXXX, XXX, XXX−XXX